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AN ASSOCIATION BETWEEN HIGH Ly-6A/E EXPRESSION ON TUMOR CELLS AND A HIGHLY MALIGNANT PHENOTYPE

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Murine Ly-6 is a molecule expressed by various cells, including several types of hematopoietic cells such as pluripotent stem cells, and activated T cells. Ly-6 is also expressed on tumor cells originating from a variety of tissues. Preliminary observations suggested that the expression of Ly-6A/E is up-regulated on highly tumorigenic variants of polyoma-virus(PyV)-transformed BALB/c 3T3 cells as compared with weakly tumorigenic variants. On the basis of these observations, we sorted PyV-transformed A3C cells or DA3 mammary adenocarcinoma cells into stable sub-populations expressing high or low levels of membrane or mRNA Ly-6A/E. *In vivo* studies indicated that the high-Ly-6A/E-expressing cells in both tumor systems expressed a considerably more malignant phenotype (higher efficiency in local tumor production as well as in lung colonization) than low-Ly-6A/E expressors. Since the high-Ly-6A/E expressors did not exhibit any growth advantage *in vitro* over low Ly-6A/E expressors, we concluded that interactions of the former cells with micro-environmental factors operating *in vivo* (*e.g.*, Ly-6A/E ligands) conferred upon these cells a highly malignant phenotype. Apart from the difference in Ly-6A/E expression, no other phenotypic characteristics distinguished highly from weakly malignant tumor cells. Similarly to T cells, where antibodies to Ly-6 transduce (or co-transduce) a proliferative signal, antibodies to Ly-6A/E were found to transduce a mitogenic signal to high-Ly-6A/E-expressing tumor cells but not to low-Ly-6A/E expressors. Taken together, these results show that Ly-6A/E expression is directly or indirectly associated *in vivo* with a highly malignant phenotype of 2 types of non-lymphoid murine tumors.

The *Ly*-6 locus in mice was first described by McKenzie *et al.* (1977). Multiple membrane antigenic specificities encoded by this locus are expressed mainly on activated T and B lymphocytes as well as on NK cells (McKenzie *et al.*, 1977; Feeney and Haemmerling, 1977; Shevach and Korty, 1989). Ly-6 mRNA or protein were also detected in kidney, heart and brain (Shevach and Korty, 1989; Van de Rijn *et al.*, 1989). Stem-cell antigen-1 (Sca-1), which belongs to the *Ly*-6 gene family, is a maker of hematopoietic stem cells in bone marrow (Van de Rijn *et al.*, 1989).

Several *Ly*-6 genes were cloned (LeClair *et al.*, 1986; Palfree *et al.*, 1988; Bothwell *et al.*, 1988; Fleming *et al.*, 1993), but the *Ly*-6 locus contains more genes (Fleming *et al.*, 1993). The *Ly*-6 locus is localized at the distal portion of chromosome 15 (LeClair *et al.*, 1987).

Ly-6 molecules are anchored to the cell membrane by glycosylphosphatidylinositol (Hammelburger *et al.*, 1987). Exposure of Ly-6-expressing cells to phospholipase C *in vitro* caused a release of such molecules to the medium (Hammelburger *et al.*, 1987). *Ly*-6 is associated with protein tyrosine kinases (Cinek and Horejsi, 1992).

Ly-6 proteins are activation molecules, especially with respect to T cells. This conclusion is based on 2 facts: the first is that Ly-6 expression is up-regulated on activated cells (Feeney and Haemmerling, 1977; Shevach and Korty, 1989); or the second is that ligation of *Ly*-6 by several monoclonal anti-Ly-6 antibodies transduces an activation or a co-activation signal manifested by increased inositol-phosphate turnover, an increase in intracellular calcium and by IL-2 production (Shevach and Korty, 1989; Malek *et al.*, 1986). Similarly to other activation molecules transducing proliferation signals to T cells, the mitogenic signal mediated by antibodies against *Ly*-6 requires the presence of the CD3-TCR complex (Yeh *et al.*, 1988; Flood *et al.*, 1990). Moreover, Ly-6 anti-sense oligonucleotides suppressed Con-A-mediated activation of T cells and inhibited the activation-associated up-regulation of Ly-6 expression of these cells (Flood *et al.*, 1990). The expression of Ly-6 proteins is regulated by interferon-α, -β and -γ (Dumont and Coker, 1986; Malek *et al.*, 1989). However, the physiological function of Ly-6 proteins and their involvement in immunological as well as other mechanisms remains unknown.

Several years ago a murine *in vitro/in vivo*-progression model system was developed in this laboratory, making it possible to identify and characterize gene products of transformed cells involved in tumor progression and controlled by host factors (Halachmi and Witz, 1989; Ran *et al.*, 1991; Gonen *et al.*, 1992; Ben Baruch *et al.*, 1992). In this model system, NIH 3T3 cells or BALB/c 3T3 cells transformed *in vitro* with c-Ha-*ras* or with polyoma virus (PyV), respectively, were cloned. These clones were either maintained in culture or passaged once *in vivo* and then returned to culture. Two types of transformed cells originating from the same clone were thus obtained: C cells (a generic term for transformed cells maintained in culture) and CTC cells (a generic term for transformed cells maintained in culture, then passaged as a tumor and returned to culture).

The most prominent, remarkable and reproducible difference between C and CTC cells is that CTC cells express a more highly tumorigenic phenotype than C cells. This was a general finding, reproducible in both transformation systems with all clones assayed (Halachmi and Witz, 1989; Gonen *et al.*, 1992). Other phenotypic characteristics may also be differentially expressed on C and CTC cells from the same clone. In general, CTC cells were found to express characteristics compatible with a more advanced position in the progression pathway (Ran *et al.*, 1991; Gonen *et al.*, 1992; Ben Baruch *et al.*, 1992).

Since Ly-6 molecules or stem-cell antigen-1 (Sca-1) are highly expressed both on proliferating cells (*e.g.*, activated lymphocytes) and on cells endowed with self-renewal potential (*e.g.*, stem cells), it was logical to examine whether highly tumorigenic CTC cells differ from weakly tumorigenic C cells in the expression of this antigen. Preliminary evidence suggested that *Ly*-6 was more highly expressed on highly tumorigenic CTC cells of PyV-transformed cells than on weakly tumorigenic C cells from the same clone (data not shown). In the present study, we examine whether the up-regulated expression of *Ly*-6 on highly tumorigenic CTC cells is indeed linked to their highly tumorigenic phenotype.

MATERIAL AND METHODS

Mice

BALB/c mice (H-2^d) were bred and maintained in the animal quarters of Tel Aviv University.

Monoclonal antibodies (MAbs)

A rat hybridoma producing anti-mouse Sca-1 (Ly-6A/E) antibody (clone E13 161-7) was obtained from the ATCC,

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Rockville, MD. Another rat anti-mouse Ly-6A/E antibody (clone D7) was obtained from Serotech, Oxford, UK. Both antibodies are of the IgG_{2a} isotype. Isotype controls were anti-Lyt-1 MAbs produced by the 53-7.313, rat hybridoma (ATCC). The mouse MAb NI/32.2, which recognizes PyV-transformed 3T3 cells, was generated in our laboratory (Ben Baruch *et al.*, 1992). A murine MAb produced by Dr. N.I. Smorodinsky of our department, which reacts with mouse astrocytes, served as an isotype (IgG_3) control for NI/32.2. The rat MAbs directed against murine FcγRII were produced by the hybridoma 2.4G2 (Ran *et al.*, 1991).

Culture media

Dulbecco's modified Eagle's medium (DMEM), FCS, penicillin, glutamine, saline containing 0.05% trypsin and 0.02% EDTA were all purchased from Biological Industries, Beit-Ha'Emek, Israel.

In vitro *growth, tumorigenicity and lung colonization of A3C and DA-3 cells*

BALB/c 3T3 cells transformed *in vitro* with PyV were cloned and maintained in culture. In this study we used C cells from the weakly tumorigenic A3 clone (A3C cells) (Halachmi and Witz, 1989). Cultured mammary adenocarcinoma DA-3 cells were derived from the D1-DMBA-3 transplantable mammary tumor (Fu *et al.*, 1990). These cells were obtained from the laboratory of I. Keydar, Department of Cell Research and Immunology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, through the courtesy of Dr. D. Lopez, Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, FL. Cultured DA-3 or A3C cells were trypsinized and washed 3 times in cold DMEM containing 10% FCS. Based on earlier tumorigenicity titration experiments of A3C cells (Halachmi and Witz, 1989) and of DA-3 cells (Carmon, Smorodinsky and Keydar, personal communication), a dose of 1×10^5 DA-3 or A3C cells per mouse was injected i.m. or s.c., respectively, into the hind leg of 8-week-old syngeneic female BALB/c mice as described (Halachmi and Witz, 1989; Fu *et al.*, 1990). Each group contained 10 mice. Spontaneous metastases were not observed.

To assess the ability of DA-3 or A3C cells to form experimental lung metastases, we inoculated single-cell suspensions of 1×10^5 cells in 0.2 ml of DMEM containing 10% FCS into the lateral tail veins of 6- to 8-week-old female BALB/c mice. Each group contained 7 mice. After 40 or 70 days, the mice were killed and the lungs were removed and weighed. Lung colonies were counted using a dissecting microscope.

Measurement of doubling time in vitro

The doubling time (dt) of *in vitro*-maintained A3C cells was calculated as described (Halachmi and Witz, 1989).

Determination of malignancy-associated phenotypes

Assays for proteolytic activity (Zymography) (Fridman *et al.*, 1992), cell adherence (Ben Baruch *et al.*, 1992), TNFα-mediated cytotoxicity (Ben Baruch *et al.*, 1992) and FcγRII expression (Ran *et al.*, 1991) were performed as described.

Flow cytometry

Cells 1×10^6 were incubated for 60 min at 4°C with the different MAbs (diluted 1:40 to 1:100). Following 2 washes with DMEM supplemented with 5% FCS and 0.05% sodium azide, the cells were incubated with FITC-conjugated secondary antibodies (goat anti-mouse IgG (Zymed, South San Francisco, CA) or rabbit anti-rat IgG (BioMakor, Rehovot, Israel)). Following 2 more washes, the pattern of antigen expression was determined using a Becton Dickinson (Mountain View, CA) FACS analyzer.

Sorting of cells according to Ly-6A/E expression

Cells 3×10^6 were incubated for 60 min at 4°C with the E13 161-7 MAb. Following 2 washes with DMEM supplemented with 5% FCS, the cells were incubated with a FITC-conjugated secondary rabbit antibody against rat IgG. After 2 more washes, cellular populations expressing high ($Ly\text{-}6^{hi}$) or low ($Ly\text{-}6^{lo}$) levels of Ly-6A/E were sorted using a FACS-IV sorter (Becton Dickinson).

Reverse transcription and PCR amplification

Total RNA was extracted from $Ly\text{-}6^{hi}$ and $Ly\text{-}6^{lo}$ A3C cells using RNAzol solution (Biotecx, Houston, TX). Two 17-base long primers, 5'GGAGTGTTACCAGTGCT3' and 5'CCACCTTGGAGCTTCTA3', were derived from positions 169 and 598 (complementary strand) of the Ly-6A/E.1 sequence (LeClair *et al.*, 1986). The PCR primers were obtained from Biotechnology General, Rehovot, Israel. Reverse-transcription reactions, following PCR amplification, were performed as described (Ochman *et al.*, 1989), using RNA originated in $Ly\text{-}6^{hi}$ and $Ly\text{-}6^{lo}$ A3C cells and the Ly-6A/E.1-derived primers described above. Reverse transcription was performed at 42°C for 1 hr, followed by 35 PCR cycles.

Statistical evaluation

Statistical analysis of the s.c. tumorigenicity assays was performed using the log-rank test (Peto *et al.*, 1977). Student's *t*-test was used to evaluate the results of lung colonization experiments and of other assays.

RESULTS

Sorting of A3C cells into stable high-Ly-6A/E- and low-Ly-6A/E-expressing cell populations

In previous studies analyzing the expression of Ly-6A/E on several PyV-transformed clones, we found that expression of this protein was generally up-regulated in highly tumorigenic CTC cells, as compared with C cells from the same clone (data not shown). This suggested a possible correlation between high *Ly*-6 expression and a highly tumorigenic phenotype. A notable exception to this rule were non-transformed BALB/c 3T3 cells. These cells, which are non-tumorigenic (*in vivo* tumorigenicity of these cells is checked periodically in our laboratory), do express Ly-6A/E (data not shown), as do other normal cells (Van de Rijn *et al.*, 1989). This indicates that Ly-6A/E expression *per se* does not confer a tumorigenicity phenotype upon non-transformed immortalized fibroblasts, but may be associated with augmented tumorigenicity in the phenotype of transformed ones.

In order to further investigate the relationship between *Ly*-6 expression on transformed cells and their tumorigenicity phenotype, we sorted C cells from the weakly tumorigenic A3 clone (A3C) (Halachmi and Witz, 1989), using the anti-Ly-6A/E (Sca-1) antibody E13 161-7 followed by a FITC-conjugated rabbit anti-rat reagent. An A3C sub-population enriched for high Ly-6A/E expression and another sub-population enriched for low-Ly-6A/E-expressing cells were grown separately in culture for a period of 2 to 4 weeks. Figure 1 shows a clear difference in the pattern of *Ly*-6 expression on these 2 cellular sub-populations. The expression of the antigen in the 2 A3C sub-populations did not change during a 6-week culture period following the reintroduction of the separately sorted cells into culture. Both sub-populations retained their high or their low Ly-6A/E expression (results not shown).

We assayed the reactivity of the high-Ly-6A/E expressors ($Ly\text{-}6^{hi}$) as well as that of the low-Ly-6A/E expressors ($Ly\text{-}6^{lo}$) with another MAb (D7) recognizing Ly-6A/E (Shevach and Korty, 1989). Figure 1 shows that cells sorted for high or low Ly-6A/E expression by the E13 161-7 antibody are correspondingly also high and low expressors for the Ly-6A/E epitopes recognized by the D7 antibody. Figure 1 also shows that both

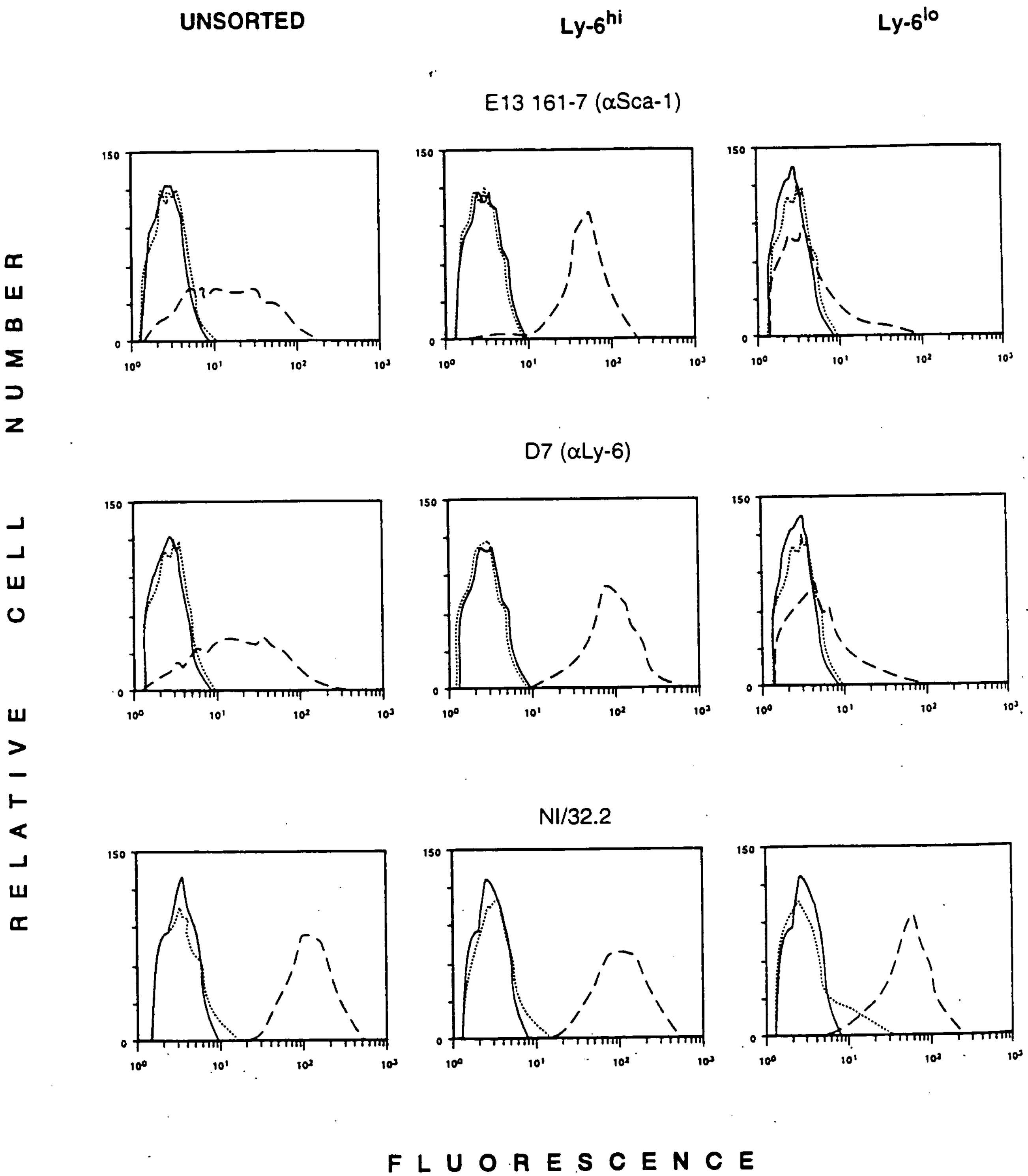

FIGURE 1 – Ly-6A/E and NI/32.2 expression by A3C sub-populations. Unsorted A3C cells and A3C cells sorted for high- or low-Ly-6A/E expression by the E13 161-7 antibody were incubated with 2 MAbs against *Ly*-6 (E13 161-7 and D7) or against NI/32.2 and then with secondary FITC-conjugated antibodies (------). Cells incubated with isotype control + secondary antibody (......) or with secondary antibodies only (———) served as controls.

Ly-6^{hi} as well as Ly-6^{lo} A3C cells expressed equal amounts of the PyV-associated NI/32.2 surface antigen (Ben Baruch *et al.*, 1992).

Reverse PCR experiments showed that Ly-6E.1 mRNA levels were much higher in Ly-6^{hi} than in Ly-6^{lo} A3C cells (Fig. 2).

These results show that a clone of PyV-transformed cells can be sorted into a sub-population enriched for high and stable Ly-6A/E expression and into a low- and stable-Ly-6A/E-expressing sub-population. The differences in Ly-6A/E ex-

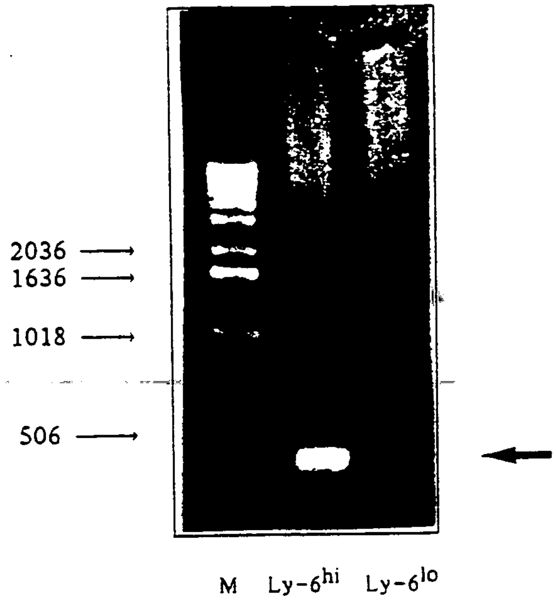

FIGURE 2 – Reverse-PCR detection of Ly-6 mRNA expression in Ly-6^{hi} and Ly-6^{lo} A3C cells. RNA prepared from Ly-6^{hi} and Ly-6^{lo} A3C cells was subjected to reverse transcription followed by PCR amplification using Ly-6E.1-derived primers. PCR amplification products were then analyzed by SDS-PAGE. The large arrow indicates the presence of the PCR reaction product. M, molecular-size (bp) markers.

pression between these sub-populations occurred both at the protein and at the mRNA levels.

Tumorigenicity of high- and low-Ly-6A/E-expressing A3C cells in BALB/c mice

The results described above indicated that *in vivo*-passaged highly tumorigenic PyV-transformed cells tend to express more Ly-6A/E than culture-maintained transformed cells expressing a weakly tumorigenic phenotype. In order to further explore a possible connection between these characteristics, we inoculated syngeneic BALB/c mice with Ly-6^{hi} or with Ly-6^{lo} A3C cells. The unsorted parental A3C population was also inoculated. Figure 3, which summarizes the results of 2 independent experiments utilizing 10 mice per treatment group in each experiment, shows that the Ly-6^{hi} cells were significantly ($p < 0.001$ by the log-rank test) more tumorigenic than the Ly-6^{lo} A3C cells or than the unsorted A3C cells. Moreover, the Ly-6^{lo} cells were significantly less tumorigenic than the unsorted parental A3C population ($p < 0.01$ by the log-rank test). It should be noted that increasing the inoculated dose of parental (unsorted) PyV-transformed cells from several clones increases tumor incidence (Halachmi and Witz, 1989; Ran *et al.*, 1991).

The level of Ly-6A/E expression by one of the 2 tumors that developed from the inoculation of Ly-6^{lo} cells was low (11% Ly-6A/E-positive cells). In contrast, 11 tumors that developed from inoculated Ly-6^{hi} cells expressed high levels of Ly-6A/E (average number of Ly-6-positive cells was 72%).

Analysis of the *in vitro* growth curves of Ly-6^{hi} and Ly-6^{lo} A3C cells showed that both types of cells have almost identical doubling times (19.25 hr *vs.* 18.76 hr for Ly-6^{hi} and Ly-6^{lo} cells respectively). This indicates that high Ly-6A/E expression *per se* does not confer a growth advantage in *in vitro* conditions, but may do so in *in vivo* conditions.

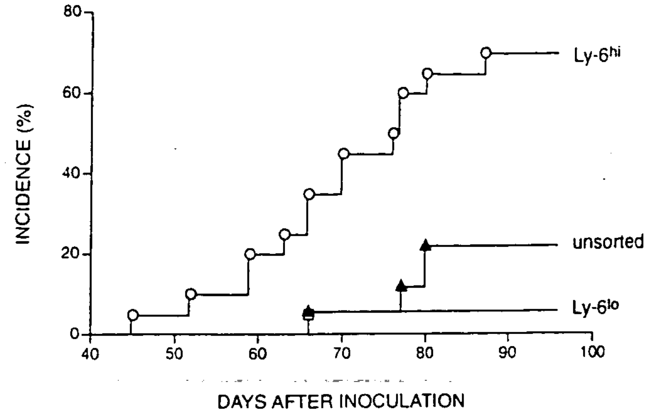

FIGURE 3 – Tumorigenicity of Ly-6^{hi} and Ly-6^{lo} A3C cells. Ly-6^{hi}, Ly-6^{lo} or unsorted A3C cells were inoculated s.c. into the right hind leg region of 10 BALB/c mice. The mice were examined for the appearance of tumors for 150 days. The figure summarizes data from 2 independent experiments utilizing 10 mice in each group in each experiment. Tumorigenicity of high-Ly-6A/E expressors was higher ($p < 0.001$ in log-rank test) than that of low-Ly-6A/E expressors. Tumorigenicity of the Ly-6^{lo} A3C cells was lower ($p < 0.01$ in log-rank test) than that of unsorted A3C cells.

Lung colonization by high- and low-Ly-6A/E-expressing A3C cells

Ly-6^{hi} and Ly-6^{lo} A3C cells as well as unsorted A3C cells were inoculated i.v. into syngeneic BALB/c mice to assess their lung colonization ability. This assay is often used to evaluate the malignancy phenotype of tumor cells (Weiss, 1983). Figure 4*a* shows the lungs of mice inoculated in 2 separate experiments with Ly-6^{hi} or with Ly-6^{lo} A3C cells. Lungs of mice inoculated with unsorted A3C cells are also shown. It is evident that Ly-6^{hi} cells have a higher ability for lung colonization than the Ly-6^{lo} sub-population or than the unsorted population of A3C cells. Quantitation of lung colonization by the 3 groups of A3C cells was done by weighing the lungs of the 3 mouse groups. Figure 4*b* shows that the lungs of Ly-6^{hi}-inoculated mice were significantly heavier ($p = 0.0001$ by Student's *t*-test) than the lungs of Ly-6^{lo}-inoculated mice, or than the lungs of mice inoculated with unsorted cells.

Tumorigenicity phenotype and lung colonization of high- and low-Ly-6A/E-expressing DA-3 mammary carcinoma cells

We examined whether the association between high Ly-6A/E expression by tumor cells and a highly malignant phenotype of these cells was limited to the PyV tumor system or whether other murine tumor cells behaved in a similar fashion.

DA-3 cells were sorted to Ly-6^{hi} and Ly-6^{lo} sub-populations, using the E13 161-7 antibody (Fig. 5). Ly-6^{hi}, Ly-6^{lo} and unsorted DA-3 cells were inoculated i.m. into syngeneic BALB/c mice. Figure 6, which summarizes the results of one experiment, shows that Ly-6^{hi} and parental unsorted DA-3 cells exhibit an identical highly tumorigenic phenotype. Ly-6^{lo} DA-3 cells, on the other hand, did not produce tumors during the duration of the experiment, which was terminated after a post-inoculation period of 42 days, when the unsorted or Ly-6^{hi} cells gave a tumor incidence of 80% or more ($p < 0.001$ log-rank). Identical results were obtained in a second *in vivo* experiment in which the tumorigenicity of Ly-6^{hi} and Ly-6^{lo} DA-3 cells was compared. This experiment was terminated 51 days after cell inoculation.

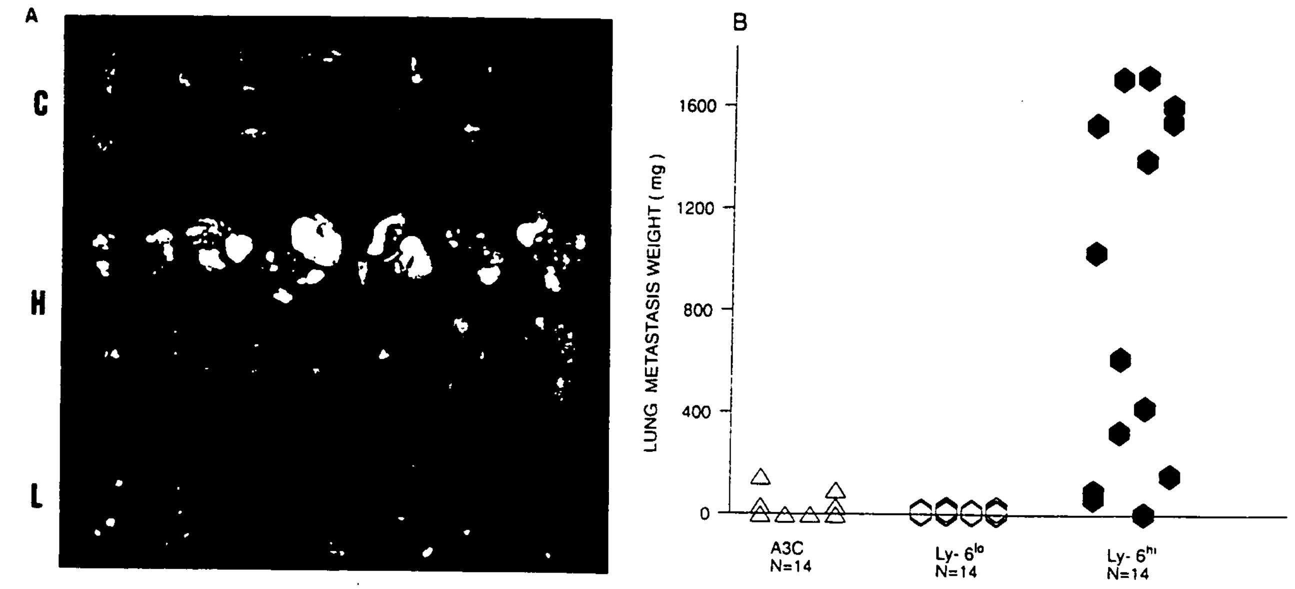

FIGURE 4 – Lung colonization by Ly-6hi and Ly-6lo A3C cells. Ly-6hi (H), Ly-6lo (L) or unsorted (C) A3C cells were inoculated i.v. into the lateral tail vein of 7 BALB/c mice. After 70 days the lungs were removed, weighed, and fixed in PBS containing 10% formalin. Each cell line was examined in 2 independent experiments. The lungs are shown in *(a)*. Weight of lung metastases *(b)*, expressed in mg, was determined by subtracting average normal lung weight (190 mg) from each lung examined.

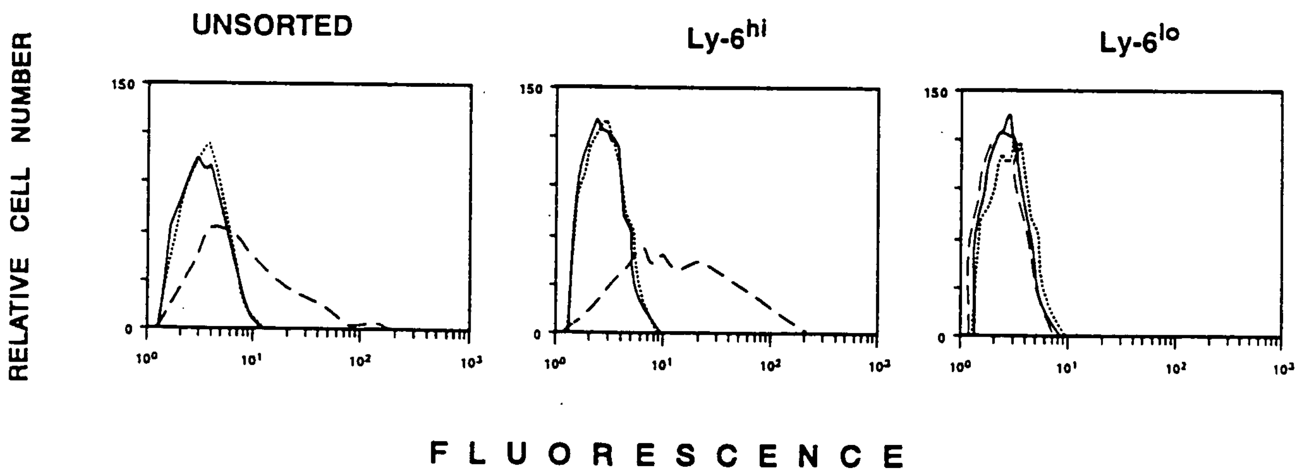

FIGURE 5 – Ly-6A/E expression by DA-3 sub-populations. Unsorted DA-3 cells and DA-3 cells sorted for high- or low-Ly-6A/E expression were incubated with the E13 161-7 MAb against *Ly-6* and then with a secondary FITC-conjugated antibody (------). Cells incubated with isotype control + secondary antibody (......) or with secondary antibody only (———) served as controls.

Although lung colonization by DA-3 cells is much lower than by A3C cells, it can be seen (Fig. 7) that the lung-colonization ability of Ly-6hi DA-3 cells is significantly higher ($p < 0.001$) than that of Ly-6lo DA-3 cells, as determined by lung weight.

Histological examination revealed carcinoma lesions in the lungs removed from mice inoculated with Ly-6hi DA-3 cells (data not shown). No pathological findings were observed in the lungs of the other experimental groups.

Like the A3C system, Ly-6hi DA-3 cells have no *in vitro* growth advantage over Ly-6lo DA-3 cells (data not shown).

Collectively, these results show that, in 2 separate tumor systems, high expression of Ly-6A/E is associated with a highly malignant phenotype of tumor cells. More important, however, is the observation that depletion of Ly-6hi tumor cells from a parental population containing a mixture of Ly-6hi and Ly-6lo cells renders the remaining Ly-6lo sub-population significantly less tumorigenic than the original population.

Anti-Ly-6A/E antibodies are mitogenic for Ly-6hi but not for Ly-6lo A3C cells

Ly-6 is an activation-transducing receptor of murine T cells whose biological ligand remains unknown thus far (Shevach and Korty, 1989). In view of the above findings, it was of interest to establish whether antibodies against Ly-6A/E which transduce proliferation signals to T cells will also do so to PyV-transformed A3C cells.

Ly-6hi A3C cells incubated for 5 days with the E13 161-7 antibody responded by increased proliferation as compared with Ly-6hi A3C cells incubated in culture medium (Fig. 8) or in the presence of a control rat antibody directed against FcγRII (data not shown). This receptor is not expressed by

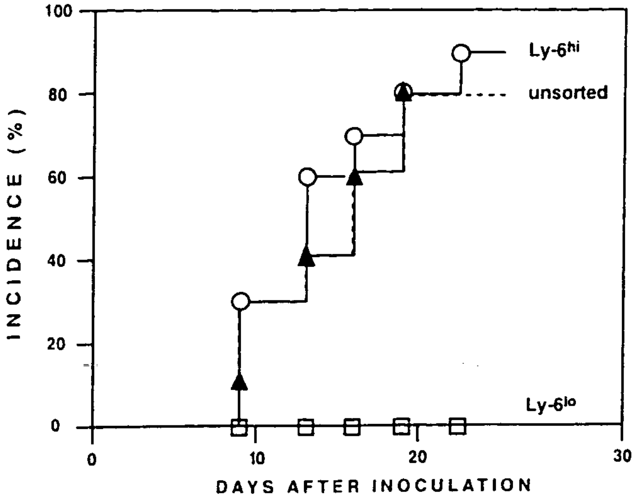

FIGURE 6 – Tumorigenicity of Ly-6^{hi} and Ly-6^{lo} DA-3 cells. Ly-6^{hi}, Ly-6^{lo} or unsorted DA-3 cells were inoculated i.m. into the hind leg of 10 BALB/c mice. The mice were examined for the appearance of tumors for a total of 42 days. Ly-6^{lo} DA-3 cells did not produce any palpable tumors during this period. Tumor incidence did not change during the period between day 22 (as shown in the figure) and the end of the experiment.

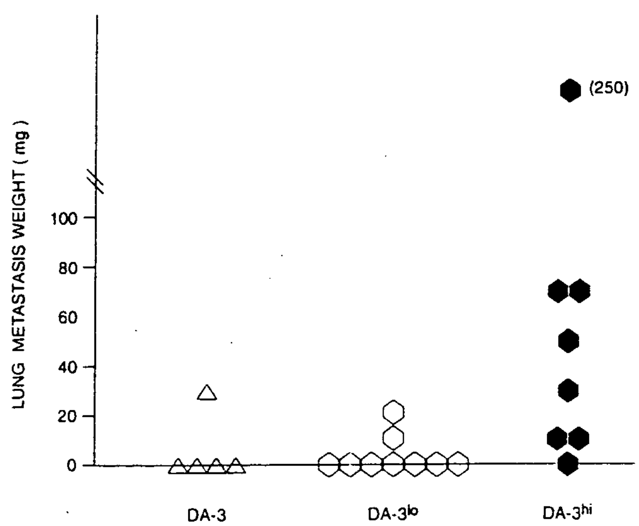

FIGURE 7 – Lung colonization by Ly-6^{hi} and Ly-6^{lo} DA-3 cells. Ly-6^{hi}, Ly-6^{lo} or unsorted DA-3 cells were inoculated into the lateral tail vein of 7 BALB/c mice. After 40 days the lungs were removed, weighed, and fixed in PBS containing 10% formalin. Weight of lung metastases, expressed in mg, was determined by subtracting average normal lung weight (190 mg) from each lung examined.

PyV-transformed C cells from several clones, including A3C cells (Ran *et al.*, 1991). The difference between the number of Ly-6^{hi} A3C cells incubated in the presence of the anti-Ly-6A/E antibody and that of the control cells incubated in culture medium or with control antibody was significant ($p < 0.05$ by Student's *t*-test). Ly-6^{lo} A3C cells were not significantly stimulated to proliferate by the anti-Ly-6 antibody. However, the somewhat elevated proliferative response of Ly-6^{lo} A3C cells to anti-Ly-6A/E antibodies can be attributed to the presence of a small number of Ly-6A/E-positive cells in this population (Fig. 1).

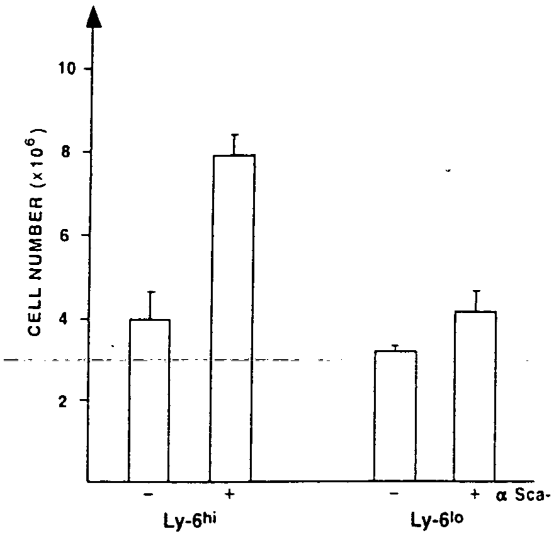

FIGURE 8 – The proliferative response of Ly-6^{hi} A3C cells to anti-Ly-6A/E MAbs. Ly-6^{hi} or Ly-6^{lo} A3C cells (2×10^4) were seeded into duplicate 25-cm^2 tissue-culture flasks and incubated with 1:10-diluted culture supernatants of the E13 161-7 anti-Ly-6, producing hybridoma (+). Controls (−) were incubated with growth medium diluted 1:10. After 7 days the cells were counted in a hemocytometer. The data are the average ±S.D. from duplicate flasks, and the experiment is one representative of 3 performed. The number of Ly-6^{hi} A3C cells incubated in the presence of the antibody was significantly higher ($p < 0.05$ as determined by Student's *t*-test) than the number of control Ly-6^{hi} A3C cells.

Phenotypic characterization of Ly-6^{hi} and Ly-6^{lo} A3C cells

The progression of PyV-transformed cells from a weakly to a highly tumorigenic phenotype may be accompanied by a series of phenotypic changes. Comparing the phenotype of highly tumorigenic CTC cells from several clones of PyV-transformed cells with that of weakly tumorigenic C cells from the same clones, we found that the former cells showed down-regulated expression of the NI/32.2 PyV-associated membrane antigen, increased resistance to TNFα-mediated killing, increased laminin-adherence (Ben Baruch *et al.*, 1992) and *de novo* acquisition of FcγRII expression (Ran *et al.*, 1991). High- and low-Ly-6A/E A3C cell sub-populations were compared with respect to the above phenotypic characteristics. Both sub-populations expressed an identical phenotype (results not shown). We also found no difference between Ly-6^{hi} and Ly-6^{lo} A3C cells with respect to their ability to secrete collagenase IV. The conclusion from these phenotype analyses is that the high-Ly-6A/E expressors do not utilize antigenic loss, increased resistance to TNF-α-mediated killing, increased laminin binding or secretion of collagenase IV as means of increasing their tumorigenicity and metastatic behavior.

DISCUSSION

Ly-6 gene products are signal-transducing molecules whose physiological function, as well as their ligands or counter-receptors, remain unknown (Shevach and Korty, 1989).

Ly-6 is expressed on various murine tumors of lymphoid or non-lymphoid origin (Shevach and Korty, 1989). In fact, several of the *Ly-6* genes were cloned from mouse tumors (Shevach and Korty, 1989). Amari *et al.* (1990) demonstrated that the Ly-6-Ril-1-Pol-5 region on murine chromosome 15 influences the susceptibility of mice to fractionated-X-irradiation-induced leukomogenesis. It was also shown by

these workers that thymomas induced by this irradiation over-express *Ly*-6, as compared with normal thymocytes. Haque *et al.* (1990) reported that a Ly-6-related protein was up-regulated on papilloma-virus-transformed rat fibroblasts. Lu *et al.* (1989) reported that administration of anti-Ly-6 MAbs to tumor-bearing mice had beneficial therapeutic effects. In view of the fact that the biological activity of the anti-Ly-6 antibodies did not correlate with *Ly*-6 expression on the tumor cells, the authors concluded that the anti-tumor activity of the injected antibodies was due to the activation of immunocytes with anti-tumor cytolytic activity via *Ly*-6 signal transduction.

This report links high expression of *Ly*-6 on non-lymphoid tumor cells with a highly malignant phenotype of cells of fibroblast (A3C) or epithelial (DA-3) origin. We do not know at this point what underlies the high malignancy phenotype of the high-Ly-6A/E-expressing tumor cells. We do know, however, that the mere expression of Ly-6A/E on such cells does not appear to confer any growth advantage, at least *in vitro*.

There are 2 major hypothetic explanations for the association between high Ly-6A/E expression and a highly malignant phenotype *in vivo*. The first postulates a cause-and-effect relationship between these 2 cellular properties. In such a case, an *in vivo* encounter between a Ly-6A/E-expressing tumor cell and a Ly-6A/E ligand or counter-receptor may possibly confer upon the *Ly*-6-expressing tumor cell a highly malignant phenotype. Non-Ly-6A/E-expressing tumor cells are obviously unable to utilize this (or another) *Ly*-6-based progression route to high malignancy. Moreover, the fact that *Ly*-6 expression does not confer any proliferative advantage upon tumor cells *in vitro* leads to the conclusion that Ly-6A/E ligands are either absent or non-functional in culture conditions. While the enhanced proliferation of Ly-6A/E-positive cells following incubation with anti-Ly-6A/E antibodies supports this supposition, there may be many other direct or indirect mechanisms underlying a causal involvement of Ly-6A/E in augmenting the malignancy of tumor cells.

The second hypothesis is that high Ly-6A/E expression and a highly malignant phenotype are causally independent. In such a case, the Ly-6A/E may be associated as a "bystander," with some unidentified property causally linked to the highly malignant phenotype of the cell. This could occur, for example, if the Ly-6A/E gene were physically or regulatorily linked to an oncogene whose up-regulated expression augments the malignancy of the tumor cells.

Tumorigenicity experiments using Ly-6^{lo} tumor cells transfected with Ly-6A/E cDNA should show definitely whether or not this gene product is causally involved in the increased malignancy of tumor cells.

Even if a non-causal association between high Ly-6A/E expression and a highly malignant phenotype is established, it is to be expected that elimination of Ly-6A/E-expressing tumor cells, for example, by immunological means, could suppress the overall malignancy of tumor-cell populations and thus serve as a novel method of cancer treatment. This expectation stems from the fact that removing Ly-6^{hi} cells from a mixed population significantly reduces the tumorigenicity of the remaining (Ly-6^{lo}) sub-population.

The broadly expressed human *CD*59 gene located on the short arm of chromosome 11 is considered by some investigators to be the human homologue of murine *Ly*-6 (Forsberg *et al.*, 1989). The 2 proteins share 26% sequence identity and have other characteristics in common: they are similar in size, and both are anchored to the cell membrane by glycosylphosphatidylinositol (Forsberg *et al.*, 1989; Stefanova *et al.*, 1989). *CD*59, like *Ly*-6 (see above), seems to function as an activation transducer in T cells (Korty *et al.*, 1991) through association with protein tyrosine kinases. *CD*2 has been identified as a ligand for *CD*59 (Hahn *et al.*, 1992). *CD*59 and related molecules are expressed by various tumor cells (Kumar *et al.*, 1993).

In view of the similarity between human *CD*59 and murine *Ly*-6, insights into the influence of *Ly*-6A/E on the malignancy phenotype of murine tumor cells may be useful in the clinical setting.

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